Recent Developments with the L5178Y TK Heterozygote Mutagen Assay System

by Donald Clive*

Introduction

The presently available mutagen test systems are no longer receiving critical acclaim by sole virtue of their positive response to ethyl methanesulfonate (EMS). Rather, they are being asked to yield answers to such questions as: how mutagenic is EMS in this system and, given this number, what does it imply relative to the genetic risk of EMS to man? Both of these questions address themselves to the relevance of the test results, the first in a quantitative manner, the second qualitatively, in that it involves evaluating species' and systems' differences. Because this extrapolation of data must be to a rather complex organism—man—we are immediately faced with a genetic quandary. Organisms which display their genetic repertoires in the simplest, most rapid manner—and which, therefore, are easiest to use and most definitive in the answers they yield—are phylogenetic foreigners to many. Used alone, one risks banning penicillin on the basis of toxic effects on gram-positive bacteria, or, to cite an example more oriented to mutagenicity testing, one might be tempted to place 5-bromodeoxyuridine (BUdR) on the GRAS list as a result of testing in thymidine kinase-deficient yeast. Yet, if we choose to eliminate most of this xenomorphism we are faced with expensive, time-consuming mega-chimp experiments which relinquish genetic secrets at such a slow rate that extrapolation to man is done in a near vacuum of supporting studies, thus weakening the advantage of evolutionary proximity.

One compromise to these extremes has been to microbialize the mammal. Growing mammalian somatic cells in tissue culture accomplishes four aims: (1) over 10^8 genetically expressive genomes are compacted into a volume less than that occupied by one average-size whole mammal; (2) the generation time—and hence the duration of the experiment—is reduced from months or years to hours; (3) definitive genetic studies, including mutagenicity studies akin to those heretofore reserved by the microbial geneticist, can now be performed on mammalian cells; and (4) we have preserved most of the structural and functional peculiarities of the mammalian genome.

However, such cells do not undergo meiosis, nor do they posses the differentiated intricacies which confer upon the whole mammal some degree of protection from a wide variety of toxic agents (but which can also activate innocuous compounds into hazardous ones). For these reasons, such *in vitro* systems do not constitute a panacea for mutagen testing. Like other systems, their limitations must be defined as ardently as their advantages are expoited.

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The $TK^{+/-}$ Heterozygote System

The advantages of mammalian somatic cells grown in tissue culture are admirably illustrated by L5178Y mouse lymphoma cells. This line of transformed cells was first isolated in the late 1950's by Fischer (*1*). They grow well in suspension culture with a generation time of 10–11 hr; they clone with 80–100% efficiency in a soft agar medium (*2,3*). We have found (*3,4*) these cells to be diploid at the thymidine kinase (TK) locus and have developed selection media for isolating TK-competent or TK-deficient mutants occurring at low frequencies among cells of the opposite phenotype. Reconstruction experiments show recovery of 80–100% of plated mutants under the conditions used. Table 1 summarizes the genetic and phenotypic characteristics of the two homozygotes ($TK^{+/+}$ and $TK^{-/-}$) and of the heterozygote ($TK^{+/-}$) clonal lines that were initially investigated (*5*). Spontaneous mutation rates, TK activities, and mode of isolation are all compatible with the assigned genotypes.

The $TK^{+/-} \rightarrow TK^{-/-}$ (our basic mutagen-assay system) mutation rate was greatly stimulated by x-irradiation, EMS, and hycanthone-methanesulfonate (*3,4,5*) (Table 2). [Hycanthone is a highly efficacious antischistosomal drug which had earlier been shown to induce frameshift mutations in *Salmonella* (*6*) and to intercalate into DNA *in vitro* (*7*)].

Table 1. Summary of phenotopic and presumed genotypic properties at the thymidine kinase (TK) locus in L5178Y mouse lymphoma cells.[a]

Spontaneous mutation rate, mutations/locus/generation	Thymidine kinase activity, pmole thymidine phosphorylated/10^6 cells per min incubation
$TK^{+/+}$ (BUdR^s)	61 ± 3.6
↓ 5×10^{-11} [b]	
$TK^{-/-}$ (BUdR^r)	0
6×10^{-9} ↙↑ 1.2×10^{-7}	
$TK^{+/-}$ (BUdR^s)	27 ± 1.3 [c]

[a] In all instances, BUdR-sensitive (BUdR^s) cells were THMG (thymidine + hypoxanthine + methotrexate + glycine)-resistant (THMG^r), and BUdR^r cells were THMG^s.

[b] One mutant only.

[c] Nine independent revertants.

Subsequent studies on a different heterozygote ($TK^{+/-}$ −3.7.2) with a higher $TK^{+/-} \rightarrow TK^{-/-}$ spontaneous mutation rate (2×10^{-6} mutations / locus / generation) show the wide range of induced mutant frequencies to which this system can respond. Figure 1 illustrates the relative mutagenicities of the three alkylating agents, EMS, MMS (methyl methanesulfonate), and EDB (ethylene dibromide) as a function of their molar

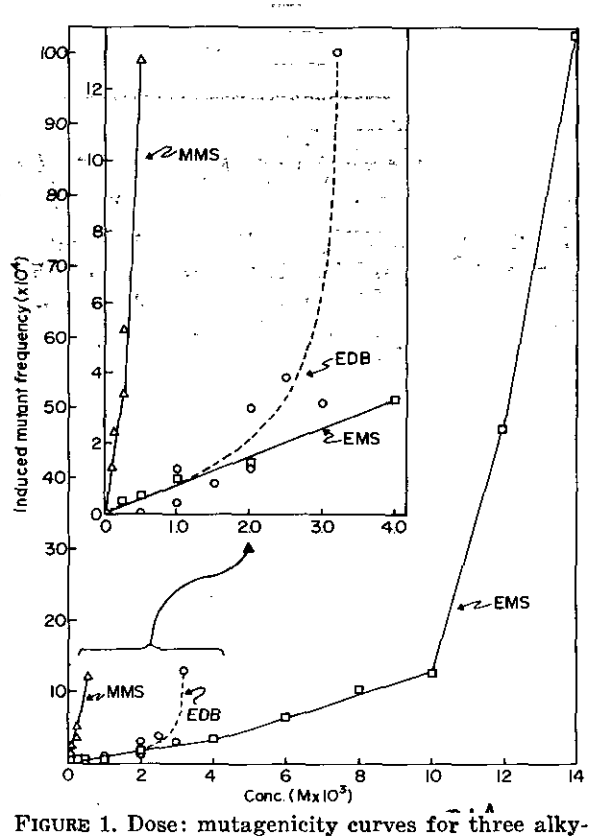

FIGURE 1. Dose: mutagenicity curves for three alkylating agents in the $TK^{+/-} \rightarrow TK^{-/-}$ mutational assay system. Induced mutant frequencies (number of BUdR-resistant ($TK^{-/-}$) mutant colonies per 10^4 viable cells) following a 2 hr exposure to the indicated mutagen concentrations (millimolar) are compared (main figure); the low dose: response range is amplified in the inset. Up to 1% mutant frequencies are inducible by EMS.

concentrations. (Induced mutant frequencies are obtained by subtracting the mutant frequency observed in the control culture from that of each of the treated cultures and in all instances is expressed in terms of number of $TK^{-/-}$ mutants per surviving cell. The vertical and horizontal scales are exaggerated in the inset to display more prominently the dose response curves of MMS and EDB. Though much less mutagenic than EMS they still represent a mutagenic equivalent of over 600 R of x-irradiation at the higher concentrations (cf. Table 2.

This system is capable of still better sensitivity, as is shown in Figure 2. Here the mutagenicity of hycanthone as a function of concentration is compared with the alkylating agents on still further exaggerated axes. The highest hycanthone concentration used is slightly more mutagenic than 600 R of x-irradiation.

So far we have accomplished little except demonstrate that the $TK^{+/-} \rightarrow TK^{-/-}$ system yields quantitative results and that different external concentrations of different mutagens effect different dose: response curves. This latter result can give seemingly contradictory results. Thus, by comparing the curves for hycanthone and EMS (Figs. 2 and 3) one can claim (a) hycanthone is about 40 times as mutagenic as EMS at $10^{-4}M$ concentration, and (b) EMS is 25 times as mutagenic as hycanthone at the highest concentrations examined. Which, if either, of these two statements is to be used when extrapolating these data to man's genetic risk, or are they

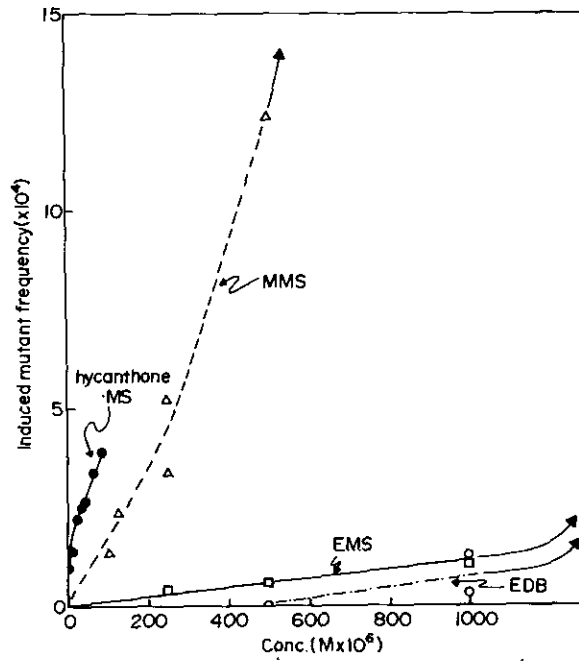

FIGURE 2. Further amplification of Figure 1, comparing dose–mutagenic response curve of hycanthone methanesulfonate with those of the alkylating agents. 600 R of x-irradiation induces approximately 3×10^{-4} mutations/locus, less than the two highest responses to hycanthone.

both relevant? What do such comparisons mean with respect to the relative efficiencies with which alkylation or intercalation events in the DNA can induce mutations?

Partial relief from such questions is obtained by replacing extracellular molar concentration of the mutagen as the independent variable in these figures by some parameter related to intracellular dose. In this manner the contribution to mutagenic response of

Table 2. Induced forward mutation rates at the thymidine kinase (TK) locus. ($TK^{+/-} \rightarrow TK^{-/-}$)

Mutagenic agent	Dose	Relative survival, %	Mutation Rate, mutations/locus/generation
None (Spontaneous)	—	100	1.2×10^{-7}
X-Irradiation	600 R (136 R/min)	12	3×10^{-4}
EMS	$10^{-2.0}M$, 2 hr	1.0	2×10^{-3}
EMS	$10^{-2.5}M$, 2 hr	10	3×10^{-4}
EMS	$10^{-3.0}M$, 2 hr	50	9×10^{-5}
Hycanthone	$1 \times 10^{-4}M$, 2 hr	2	5.4×10^{-5}
Hycanthone	$0.5 \times 10^{-4}M$, 2 hr	20	3.4×10^{-5}
Hycanthone	$0.2 \times 10^{-4}M$, 2 hr	55	1.0×10^{-5}

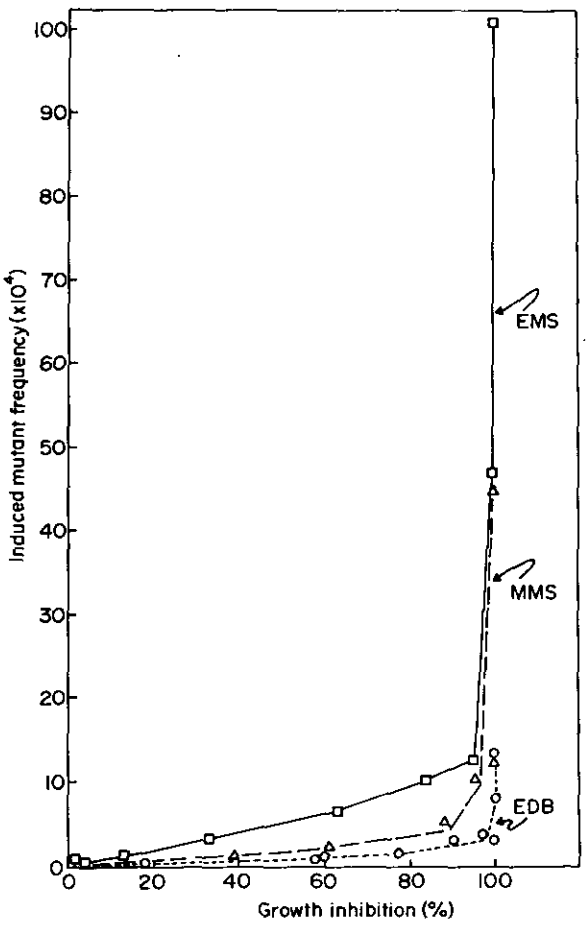

FIGURE 3. Mutant frequencies (per 10^4 viable cells) induced by three alkylating agents, expressed as a function of growth inhibition. The curves of Figures 1 and 2 are made to overlap in their abscissal range by relating mutagenic response to a biological (growth inhibition), rather than to a chemical (molarity) measure.

differences in membrane permeability to and/or transport of various mutagens would be minimized. Ideally, this should be number of mutagen molecules physically associated with the TK gene, but except for EMS (see below) such dosimetric relationships have not yet been established. Instead, we have chosen growth inhibition as a more easily assessible index of intracellular—and possible genomal—concentration.

Growth inhibition is measured in two steps. First, the rate of growth in suspension culture is determined for each treated culture for the combined treatment (2 hr) and expression (48–72 hr) times (sham-treated control = 100). Then cloning efficiencies (relative to Coulter counts) are assessed for each culture (control = 1.00). The product of these two figures for each culture represents the overall growth of that culture relative to the control (= 100%); growth inhibition is obtained by subtracting these relative growth figures from 100.

The previous data can now be plotted over a common abscissal range (0–100% growth inhibition) as in Figures 3 and 4. Note (Fig. 4) that EMS appears to be a much more potent mutagen over the whole range of growth inhibitions covered, while MMS, hycanthone, and possibly even EDB fall fairly closely together.

These data are now in a format which is both quantitative and easily compared with

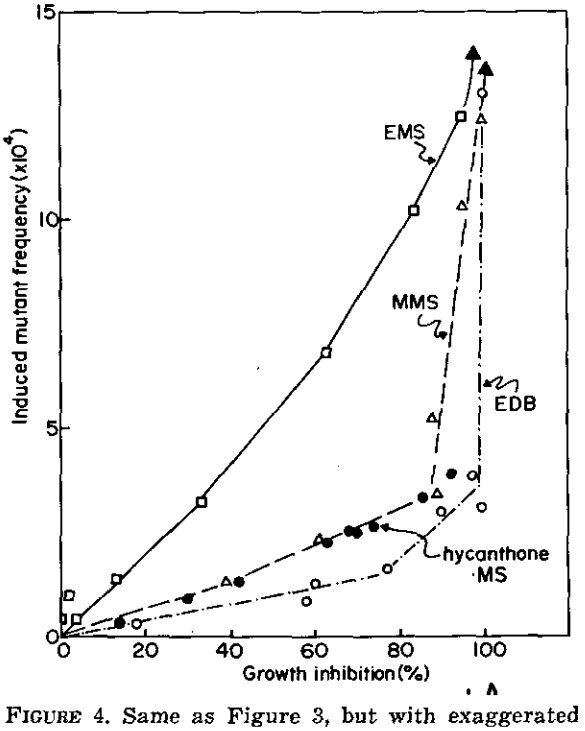

FIGURE 4. Same as Figure 3, but with exaggerated vertical axis and inclusion of hycanthone data. Note the relative high induction of mutations per lethal event (slope of curve) for EMS, and the essential similarity of this index for MMS, EDB and hycanthone, despite qualitatively different mutagenic mechanisms (alkylation and base pair substitutions vs. intercalation and frame-shifts) involved.

other systems. If such comparisons show that the mutagenicity—toxicity relationships for a given compound are not too highly species-dependent—or, if they are, and if well-established biochemical or physical difference in the more xenomorphic systems can adequately explain such differences—then the data can be directly extrapolated to man and an estimate of the genetic risk can be hazarded. The emphasis is, of course, on high correlations among various test systems after allowing for their respective pitfalls.

As a beginning in putting our mutagenicity test systems to positive social advantage we have been studying the mutagenicity of a number of hycanthone analogs possessing varying degrees of antischistosomal activity. The mutagenicity—or lack thereof—of one of these, IA–3 (the 6-chloroindazole derivative of lucanthone), is compared with that of hycanthone in Figure 5 as a function of both molar concentration and growth inhibition. At up to 80% inhibition of growth this compound is no more than 10% as mutagenic as hycanthone. Of course, further testing in other systems is essential before drawing firm conclusions relative to clinical potential. Especially, the question of whether hydroxylation of the 4-methyl group occurs in whole mammals (as it does for lucanthone) must be considered, since the hydroxymethyl derivative of IA–3 is 3 times as mutagenic as IA–3 itself (i.e., it is 30% as mutagenic as hycanthone).

As alluded to above, these cells readily lend themselves to dosimetry studies at the genomal level. Specifically, Patterson (*8*) has determined the extent of ethylation of the DNA over a tenfold range in EMS concentration; by comparing these data with the EMS mutagenicity data an estimated 8 $\times$ 10^{-5} mutations per alkylation occur which is constant over a 5-fold concentration range (see Fig. 6). Allowance must still be made for the unknown fraction of all possible mutations at the TK locus which are not amorphic and hence not detectable

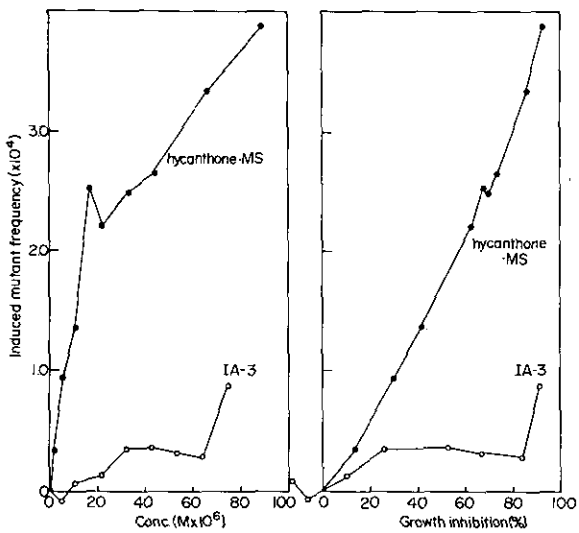

FIGURE 5. Mutagenicity of the 6-chloro-4-methylindazole derivative (IA–3) of hycanthone, relative to that of hycanthone. Considered as a function of either concentration or growth inhibition this compound is nearly nonmutagenic in this system.

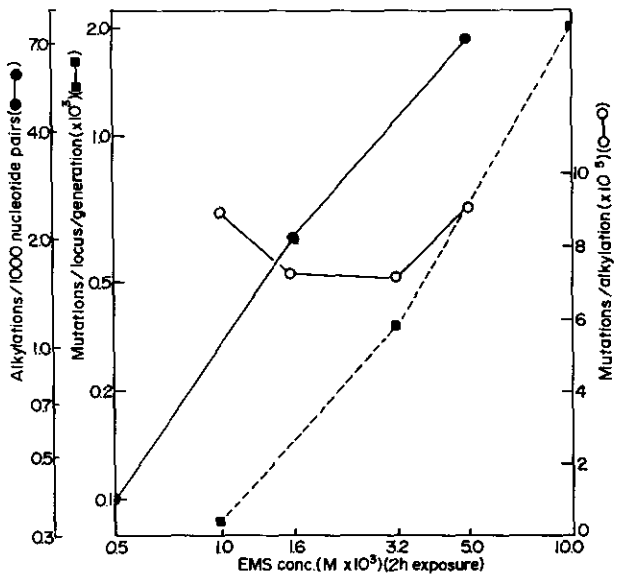

FIGURE 6. Dosimetry of EMS in the $TK^{+/-} \longrightarrow TK^{-/-}$ mutation assay system: (●) extent of alkylation of the DNA was determined following a 2 hr exposure to [^{3}H]–EMS (constant radioactivity over indicated EMS range); (■) induced mutant frequencies were measured as usual following the standard 2 hr exposure; (○) the ratio, number of mutations/alkylation, is essentially constant at 0.8 $\times$ 10^{-4} over a 5-fold variation of EMS concentration (1.0–5.0 *mM*).

in this system. Even assuming as little as 1% of all TK mutants to be amorphic, we still have a figure of approximately 1 mutation per 100 alkylations, raising the possibility of a 99% level of repair efficiency.

It would appear from the foregoing that the $TK^{+/-} \rightarrow TK^{-/-}$ L5178Y mouse lymphoma mutagen assay system is (1) faster than a breeding mammal; (2) able to eke much data from a simple experiment; (3) more relevant than *Salmonella;* and (4) able to distinguish quantitatively among the mutagenicities of closely related analogs (e.g., EMS, MMS; hycanthone, IA–3). Additionally, an attempt has been made to present these data in a form which encourages comparison with other systems (since toxicity is an easily quantitated parameter) and of extrapolation to man. Weaknesses do exist and these have been briefly touched upon. But some of these weaknesses might be surmountable. Certainly the absence of host metabolism can be at least partially overcome by coupling the test system as described to a microsomal activation system such as that described by Malling and Frantz (*9*) or by utilizing these cells as indicator organisms in a host-mediated assay, as discussed by Capizzi (*10*) and Lee (*11*).

Conclusions

The mammalian cell culture system just described helps bridge the gulf between microbial—especially prokaryotic—systems and whole mammal systems presently in use for mutagenicity studies. Such a claim follows from the fact that these lymphoma cells retain both a typically mammalian architecture (down to and including the genome) and certain facets of mammalian metabolism. Quantitative aspects of mutagenesis have been emphasized and mutagens can be classified as strong, weak, or of intermediate potency on the basis of their mutagenicity: growth inhibition curves. In an attempt to improve upon growth inhibition as a dosimetric measure, we have determined the relationship between extent of EMS-induced alkylation and resultant mutant frequency, and plan on extending such studies to other mutagens. Finally, a number of hycanthone analogs are being studied; one of these has been shown to exert possibly negligible mutagenicity at concentrations which are highly inhibitory to growth. It is hoped that such studies can lead to a safe hycanthone substitute for the control of schistosomiasis.

This workshop has demonstrated that basic agreement presently exists on the desirability of multiple test systems; multiple test systems exist, each with their characteristic strengths and weaknesses, for screening and safety testing programs; a possible hierarchy of such programs has been described by Bridges (*12*). We can move on to the real world where mutagens do not attack in single file but *en masse;* where the target cells are not intraperitoneal, but gonadal; where the issue is not only dead fetuses, but an imperceptibly debilitating population; and where *Salmonella sapiens* does not exist.

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